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Stealth Proteins: In Silico Identification of a Novel Protein Family Rendering Bacterial Pathogens Invisible to Host Immune Defense

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There are a variety of bacterial defense strategies to survive in a hostile environment. Generation of extracellular polysaccharides has proved to be a simple but effective strategy against the host's innate immune system. A comparative genomics approach led us to identify a new protein family termed Stealth, most likely involved in the synthesis of extracellular polysaccharides. This protein family is characterized by a series of domains conserved across phylogeny from bacteria to eukaryotes. In bacteria, Stealth (previously characterized as SacB, XcbA, or WefC) is encoded by subsets of strains mainly colonizing multicellular organisms, with evidence for a protective effect against the host innate immune defense. More specifically, integrating all the available information about Stealth proteins in bacteria, we propose that Stealth is a D-hexose-1-phosphoryl transferase involved in the synthesis of polysaccharides. In the animal kingdom, Stealth is strongly conserved across evolution from social amoebas to simple and complex multicellular organisms, such as *Dictyostelium discoideum*, hydra, and human. Based on the occurrence of Stealth in most Eukaryotes and a subset of Prokaryotes together with its potential role in extracellular polysaccharide synthesis, we propose that metazoan Stealth functions to regulate the innate immune system. Moreover, there is good reason to speculate that the acquisition and spread of Stealth could be responsible for future epidemic outbreaks of infectious diseases caused by a large variety of eubacterial pathogens. Our in silico identification of a homologous protein in the human host will help to elucidate the causes of Stealth-dependent virulence. At a more basic level, the characterization of the molecular and cellular function of Stealth proteins may shed light on fundamental mechanisms of innate immune defense against microbial invasion.

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Introduction

Colonization of hosts by microorganisms is a complex process that determines if the microorganism will coexist with the host as commensal, become an invasive pathogen, or be efficiently eliminated by the host's immune defense [1,2]. Consequently, microorganisms have developed a variety of measures to cope with the increasingly sophisticated defense strategies of the host's immune system [3–7]. Amongst them, the generation of an extracellular coat made of polysaccharides has proved to be a simple but effective strategy. Bacterial surface polysaccharides can be either amorphous exopolysaccharides, anchored in the lipid layer (lipopolysaccharides, another known regulator of the immune system), or organized as a capsule (capsule polysaccharides [CPSs]). The latter have been shown to mediate adherence to cells and, more importantly, protection against the host's innate immune system [8–11].

Different strategies to escape host immune surveillance have evolved through vertical evolution but also through horizontal gene transfer [12–15]. Though a subject of long-standing controversy, there is increasing evidence suggesting that horizontal gene transfer also occurs from eukaryotes to prokaryotes [16]. Even though the recombined bacteria seemed to have preferentially retained individual domains of proteins [16], a first example was recently reported in which certain bacterial strains kept an entire open reading frame [17].

Here we describe a novel protein family named "Stealth." Based on a comparative genomics approach, we propose a biological function and an evolutionary scenario for this new protein family.

Results/Discussion

Identification of Stealth

In a screen of the human genome for Notch-related proteins, a novel protein containing two copies of Lin-12/Notch repeats was identified. The protein also showed strong sequence similarity to a number of animal and bacterial proteins, including several virulence factors of human pathogens published under different names. This previously unknown protein family was named "Stealth" because experimentally characterized members of this family appear

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Abbreviations: CPS, capsule polysaccharide; CR, conserved region

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Synopsis

The immune system is a complex and highly developed system of specialized cells and organs that protects an organism against bacterial, parasitic, fungal, and viral infections. Broadly speaking, the different types of immune responses subdivide the immune system into two categories: innate (or nonadaptive) and adaptive immune system. The innate immune system serves as a first line of defense but lacks the ability to recognize certain pathogens and to provide the specific protective immunity that prevents reinfection. Just as metazoans have developed many different defenses against pathogens, so have pathogens evolved elaborate strategies to evade these defenses. Based on a comparative genomics approach and data mining, the authors have discovered a new family of proteins with a striking phylogenetic distribution, occurring in most eukaryotes and in subsets of mostly pathogenic or commensal prokaryotes. While the precise functions of these proteins remain unknown, prokaryotic versions have been implicated in the synthesis of extracellular polysaccharides known to be potent regulators of the innate immune system. This previously unrecognized link hints towards a potentially novel regulatory mechanism of the innate immune system. It remains to be shown if drugs selectively inhibiting Stealth in pathogens will help fight Stealth-mediated infections.

to render bacterial and protozoan invaders invisible to the host's immune surveillance system.

Stealth proteins are characterized by four conserved regions (CRs) referred to as CR1 to CR4 (Figure 1). The N-terminal CR1 consists of a short but strongly conserved sequence motif, IDVVYTF or very similar. The second region, CR2, is approximately 100 residues long and constitutes the most conserved part of this protein family. A standard BLAST search [18] with any CR2 domain identifies all other members of the Stealth family in the current database with highly significant *E*-values. CR3 is about 50 residues long but less well conserved. Finally, the C-terminal CR4 includes an almost universally conserved tetrapetide, CLND or CIND. Adjacent and between these domains are divergent sequence regions of variable length that may contain additional domains (Figures 1 and 2A).

Taxonomic Distribution

Stealth proteins are found encoded in the genomes of chordates, echinodermates, hydras, fungi, and flies but appear to be absent from nematodes and plants. Interestingly, a few organisms contain multiple Stealth genes (Table 1). Stealth proteins also occur in the protist genomes of *Dictyostelium, Giardia, Leishmania, Entamoeba,* and *Phytophthora,* and among the hitherto sequenced bacteria, they are found in the following phyla: alpha-, beta-, and gamma-proteobacteria (mostly pathogens), firmicutes (mostly the commensals), and actinobacteria (some animal pathogens) (Table 1; Figure S1). It is noteworthy that the large majority of completely sequenced bacterial genomes do not harbor Stealth. The species that do contain a member of this family are not necessarily closely related, and include Gram-positive as well as Gram-negative bacteria.

Stealth in Bacteria

Several of the documented bacterial Stealth genes belong to capsule group II biosynthesis operons generating carbohydrate-phosphodiester-containing CPSs [19–24]. In the case of Stealth-expressing bacteria, these CPSs turned out to inhibit complement-mediated lysis, as shown for serogroup A and X of *Neisseria meningitidis* [23,24] and to correlate with serum and phagocyte survival abilities as shown for *Aeromonas hydrophila* [25].

The majority of Stealth-expressing bacteria that have been analyzed so far for the composition of their exopolysaccharides turned out to build phosphoglycans consisting of phosphodiester-linked hexose mono- or disaccharide building blocks [26–29]. On the other hand, certain bacteria living in a biofilm community contain CPSs consisting of phosphodiester-linked hexa- or heptasaccharide repeating units [30,31]. These carbohydrates, also called receptor polysaccharides, are synthesized by a series of different glycosyltransferases, with Stealth amongst them [22]. Strains encoding Stealth carry a hexose phosphodiester linker [31] in their receptor polysaccharides, whereas strains lacking Stealth build receptor polysaccharides with a pentose phosphodiester linker.

Definite proof for an essential function of Stealth in CPS biosynthesis was shown in *N. meningitidis* serogroup A by selective deletion of the gene *sacB* (i.e., Stealth), giving rise to virtually unencapsulated mutants [23], and by deletion of part of the gene *xcbA* (i.e., Stealth), together with flanking open reading frames in a serogroup X strain, which resulted in complement-sensitive mutants [24]. Moreover, when the gene *cps1A* (i.e., Stealth) was deleted in *Actinobacillus pleuropneumoniae,* the resulting strains lost their pathogenicity in pigs [20].

Taken together, all of the above data suggest that Stealth is a D-hexose-1-phosphoryl transferase that generates interglycosidic phosphate diester linkages.

Characteristics of Metazoan Stealth

Unlike the bacterial Stealth proteins, the vertebrate members of this family are not properly represented in current protein databases. We have manually reconstructed the gene and protein sequences for a number of species with the aid of EST sequences and cross-genome comparisons (Table 1). The human gene consists of 21 exons (Figure 2B), and the translated protein sequence is identical to the RefSeq entry NP__077288. The intron–exon structures of genes found in other vertebrates are essentially the same. In the mouse, however, there is a facultative intron near the start codon spliced out predominantly in transcripts from dendritic cells. This alternative splicing leads to two protein variants with different N-termini (Figure 2C). The hypothetical *Drosophila melanogaster* and *D. yakuba* Stealth genes, however, have a completely different intron–exon structure (Figure 2B). Finally, pieces of Stealth-encoding sequences were also found in the preliminary genomes or ESTs of other mammals (Table 1).

Metazoan Stealth proteins are characterized by additional domains. There is a predicted signal peptide and, near the C-terminus, a transmembrane helix. One or two Notch/Lin-12 repeats [32] are inserted between CR2 and CR3, and an EF-hand domain [33] appears between CR3 and CR4. So far, all reconstructed Stealth proteins contain these domains, and in some of the cases where only pieces of sequences are available one can identify these motifs. The strong conservation of the Stealth domain architecture suggests that this protein plays an essential role.

No experimental knowledge is available about the function of metazoan Stealth proteins today (note, however, that

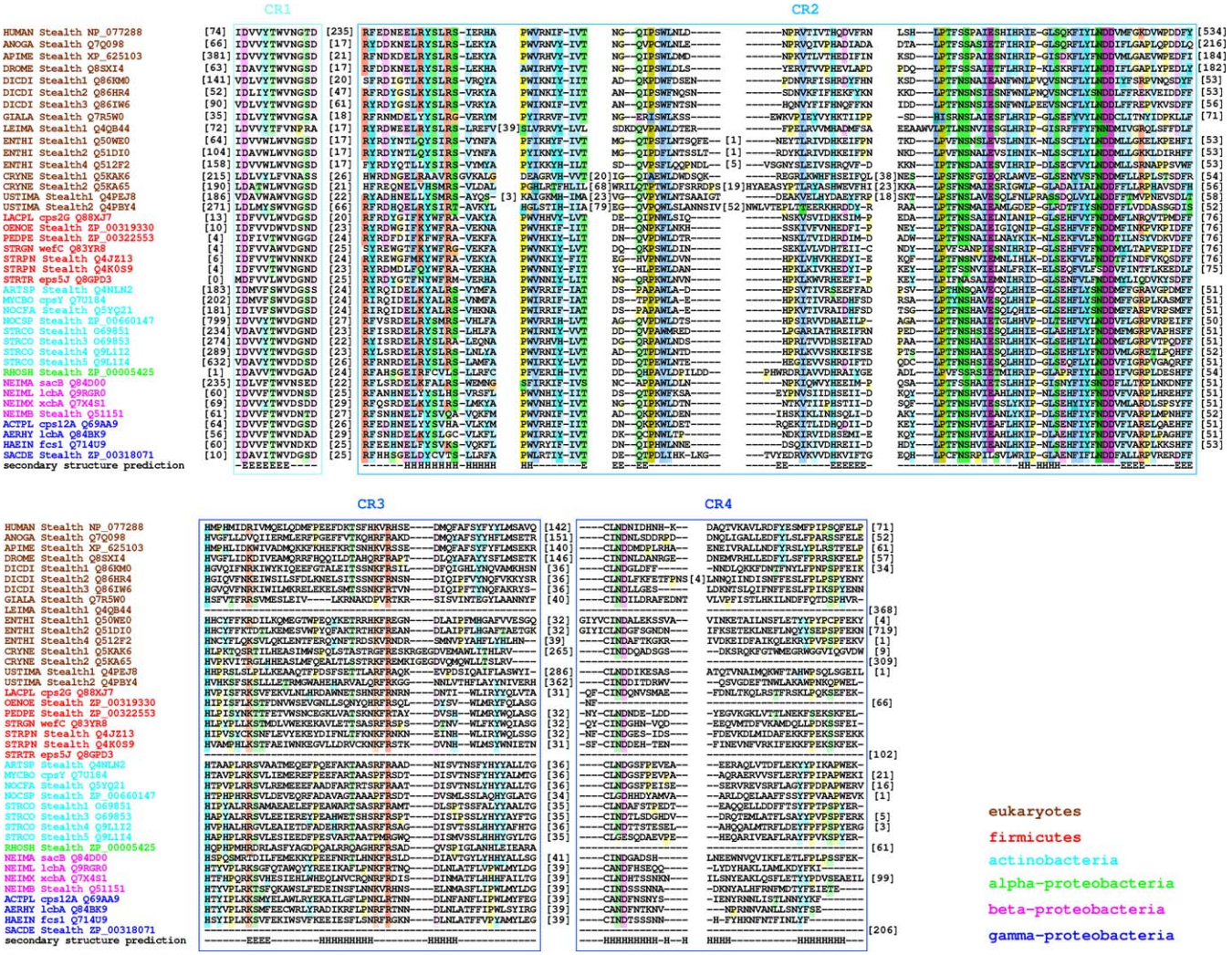

Figure 1. Multiple Alignments of CRs

Multiple alignments of the four CRs for a representative set of protein sequences (>15% dissimilarity over all four CRs) are shown. Sequences are identified by a species code (see Table 1), protein name (from literature as proposed in this paper), and database accession number, where available. The lengths of the sequences omitted between or within CRs are indicated in square brackets. The last row shows the secondary structure prediction obtained by jnetpred [65] for the human Stealth protein, where H stands for helices and E for beta-sheets. The color scheme used is the ClustalX default scheme, with the colors for conserved amino acids being more intense than those for nonconserved ones.

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Stealth-deficient mice have been generated by O. Z. and coworkers and will be made available upon request). In view of the high degree of sequence similarity to their bacterial homologs, it is reasonable to speculate that they have a similar molecular function and thus are also implicated in exopolysaccharide synthesis. Public expression profiles derived from SAGE experiments indicate a rather broad tissue distribution. The Stealth-dependent polysaccharides could be host-specific structural surface elements exploited by the immune system for self-recognition. In this case, the Stealth-dependent resistance of human pathogens to complement-mediated lysis and other host defense mechanisms would be a straightforward case of molecular mimicry. Alternatively, host-encoded Stealth proteins may play an active role in down-regulating the immune response. The presence of Stealth in both insects and urochordates further suggests that this protein interferes with processes related to innate rather than adaptive immunity [34,35].

Figure 2. Domain Architecture and Genome Structure

(A) CR1 to CR4, found through multiple alignments, are represented by rectangles ranging from light blue (CR1) to dark blue (CR4). Other motifs are represented as follows: predicted signal peptides as magenta rectangles, transmembrane regions as orange rectangles, Lin-12/Notch repeats as red pentagons, and EF-hands as green circles.

(B) The genome structure of the human and fly Stealth homologs is represented, with the exons depicted as green rectangles separated by introns of indicated size.

(C) Two splice variants lead to different N-terminal sequences, as supported by mouse EST sequences. Splicing reconstructs a codon for tyrosine (Y). Both proteins contain a predicted signal peptide.

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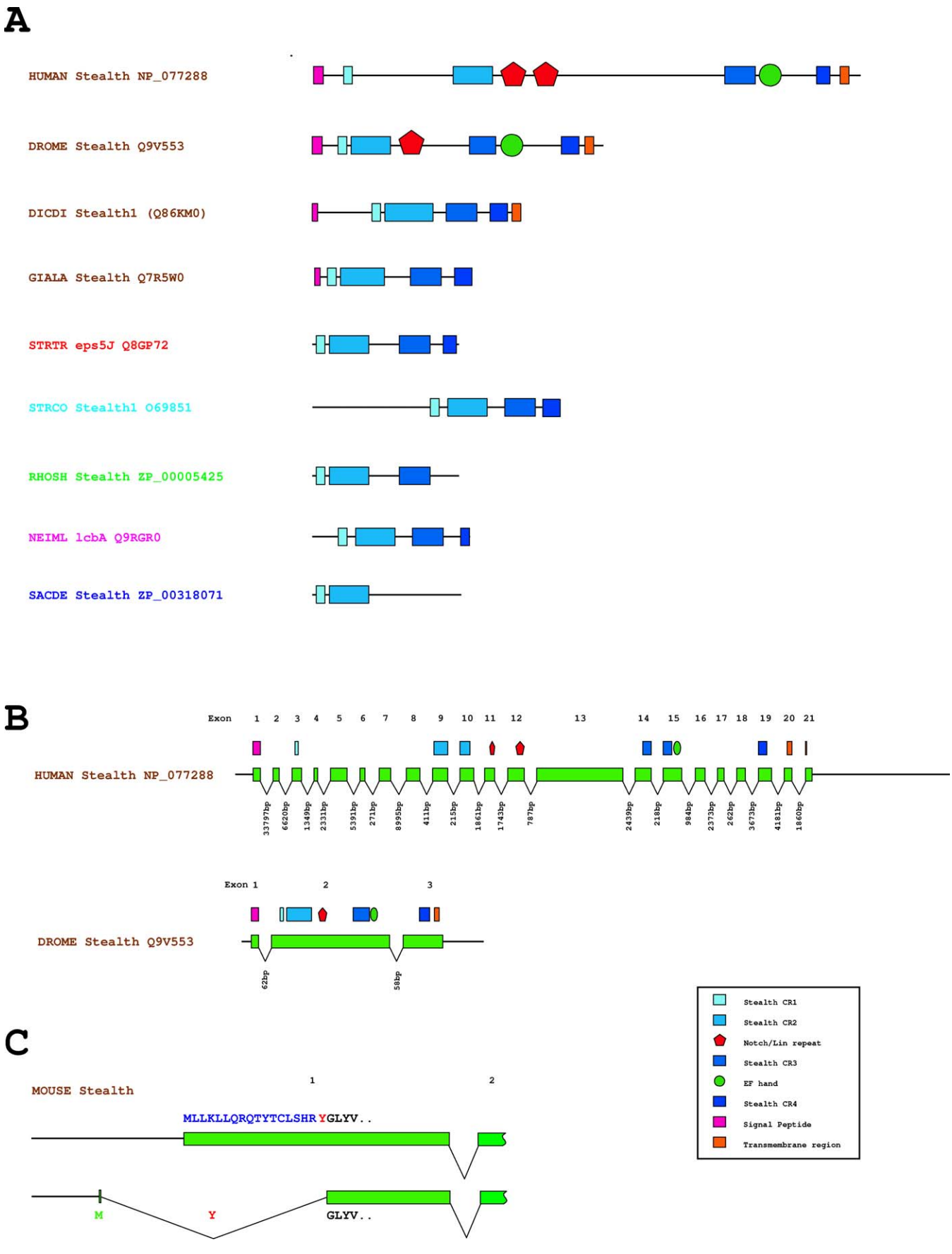
A
HUMAN Stealth NP_077288
DROME Stealth Q9V553
DICDI Stealth1 (Q86KM0)
GIALA Stealth Q7R5W0
STRTR eps5J Q8GP72
STRCO Stealth1 O69851
RHOSH Stealth ZP_00005425
NEIML lcbA Q9RGR0
SACDE Stealth ZP_00318071
B
Exon 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
HUMAN Stealth NP_077288
3797bp 6620bp 1349bp 2331bp 5391bp 271bp 8995bp 411bp 215bp 1861bp 1743bp 787bp 2439bp 218bp 984bp 2373bp 262bp 3673bp 4181bp 1860bp
Exon 1 2 3
DROME Stealth Q9V553
62bp 58bp
Stealth CR1
Stealth CR2
Notch/Lin repeat
Stealth CR3
EF hand
Stealth CR4
Signal Peptide
Transmembrane region
C
MOUSE Stealth
1 2
MLLKLLQRQTYTCLSHRYGLYV..
M Y GLYV..

Table 1. Summary of All Species Containing Stealth Proteins

Taxon	Species	Code	Strain	ID[a]	Gene
Actinobacteria	*Arthrobacter* sp.	ARTSP	FB24	Q4NLN2	NA
	Mycobacterium leprae	MYCLE	TN	Q50025	*cpsY*
	Mycobacterium tuberculosis	MYCTU	H37Rv (CDC1551)	O06628 (Q7D992)	*cpsY*
	Mycobacterium bovis	MYCBO	AF2122/97	Q7U184	*cpsY*
	Nocardia farcinica	NOCFA	IFM 10152	Q5YQ21	NA
	Nocardioides sp.	NOCSP	JS614	ZP_00660147 (GI:71369725)	NA
	Streptomyces coelicolor	STRCO	A3(2)/M145	O69851, O69852, O69853, Q9L1I2, Q9L1I4	SC1C3.09–11, SCC88.05c-03c
Alpha-proteobacteria	*Rhodobacter sphaeroides*	RHOSH	36983	ZP_00005425 (GI:46193085)	NA
Beta-proteobacteria	*Neisseria meningitidis* serotype A	NEIMA	Z2491, M2677, M4775, M1124, F8229	Q9JWW8, Q84CZ9, Q84D00, Q83U59, O68215	*sacB*
	Neisseria meningitidis serotype L	NEIML	NA	Q9RGR0	*lcbA*
	Neisseria meningitidis serotype X	NEIMX	M7575	Q7X4S1	*xcbA*
	Neisseria meningitidis serotype B	NEIMB	NA	Q51151	NA
Gamma-proteobacteria	*Actinobacillus pleuropneumoniae*	ACTPL	4074, 8329	Q8KSB4, Q69AA9	*cps1A, cps12A*
	Actinobacillus suis	ACTSU	SO4	Q84CH1	NA
	Aeromonas hydrophila	AERHY	PPD 11/90, JCM3983	Q84BK9, Q848R7	*lcbA*
	Haemophilus influenzae	HAEIN	700222	Q714U9	*fcs1*
	Saccharophagus degradans	SACDE	14642	ZP_00318071 (GI:48864178)	NA
Firmicutes	*Lactobacillus plantarum*	LACPL	NCIMB8826/WCFS1	Q88XJ7	*cps2G*
	Oenococcus oeni	OENOE	PSU-1	ZP_00319330 (GI:48865470)	NA
	Pediococcus pentosaceus	PEDPE	ATCC 25745	ZP_00322553 (GI:48869813)	NA
	Streptococcus gordonii	STRGN	38	Q83YR8	*wefC*
	Streptococcus mitis	STRMI	NCTC 12261	Q6L5Q5	*wefF*
	Streptococcus sobrinus	STRSO	6715	TIGR_246202	NA
	Streptococcus pneumoniae	STRPN	546/62, 34365, Sutcliff, Colemore, Johnson	Q4K0R3, Q512F2, Q4K2S1, Q4JZ13, Q4K2U1	NA
	Streptococcus oralis	STROR	NA	Q6L5S6	*wefC*
	Streptococcus thermophilus	STRTR	NCFB 2393	Q9EVX1	*cpsJ*
	Streptococcus thermophilus eps type V	STRTR	NA	Q8GPD3	*eps5J*
	Streptococcus thermophilus eps type IX	STRTR	NA	Pseudogene	*eps9J* (pseudogene)
	Streptococcus thermophilus eps type X	STRTR	NA	Q8GP72	*eps10H* (*eps10N* pseudogene)
Eukaryotes	*Anopheles gambiae*	ANOGA	PEST	Q7Q098 (Chromosome 3L, MOZ2, NCBI build 2.2)	NA
	Apis mellifera	APIME	DH4	XP_625103 (unknown chromosome, Amel v2.0)	NA
	Bos taurus	BOVIN	NA	Contigs 130770, 99679, 39654 (draft genome v1.0)	NA
	Canis familiaris	CANFA	NA	Chromosome 15 (CanFam1.0, NCBI build 1.1)	NA
	Ciona intestinalis	CIOIN	NA	Scaffold 341 (unknown chromosome, draft genome v1.95)	NA
	Cryptococcus neoformans	CRYNE	B-3501A (JEC21)	Q55KX3 (Q5KAK6) (Chromosome 10, NCBI build 1.1), Q55LC0 (Q5KA65) (Chromosome 10, NCBI build 1.1)	NA
	Cyprinus carpio	CYPCA	NA	EST: CF660934	NA
	Brachydanio rerio	BRARE		Q5RGJ8 (Chromosome 4, NCBI build Zv4)	NA
	Dictyostelium discoideum	DICDI	AX4	Q86HR4, Q86KM0, Q86IW6 (Chromosome 2)	NA
	Drosophila melanogaster	DROME	NA	Q9V553 (Q8SXI4) (Chromosme 2L, NCBI build 4.1)	NA
	Drosophila pseudoobscura	DROPS	MV2–25	EAL25704 (GI:54636301) (unknown chromosome, draft genome release 1)	NA
	Drosophila yakuba	DROYA	NA	chromosome 2L (April 2004 freeze)	NA
	Entamoeba histolytica	ENTHI	HM-1:IMSS	Q50WE0, Q51DI0, Q50UW0, Q512F2, Q51GM7	NA
	Fugu rubripes	FUGRU	NA	Prediction based on assembly version 3.0	NA
	Fundulus heteroclitus	FUNHE	NA	EST: CN983537, CN953211, CN972229, CN957210	NA
	Gallus gallus	CHICK	NA	Chromosome 1 (prediction based on February 2004 freeze)	NA
	Giardia lamblia	GIALA	WBC6	Q7R5W0	NA
	Halocynthia roretzi	HALRO	NA	EST: AV382587	NA
	Haplochromis chilotes	HAPCH	NA	EST: BJ674470	NA
	Homo sapiens	HUMAN	NA	NP_077288 (GI:38202211) (Chromosome 12, NCBI build 35.1)	NA
	Hydra magnipapillata	HYDMA	NA	EST: CN560699, CN560453	NA
	Ictalurus punctatus	ICTPU	NA	EST: BM495651, BM495632, BM496752	NA
	Leishmania major	LEIMA	Friedlin	Q4QB44 (Chromosome 23), Q4QB45 (Chromosome 23)	NA
	Mus musculus	MOUSE	NA	BAD32410 (GI:50510849) (Chromosome 10, NCBI build 34.1)	NA
	Oncorhynchus mykiss	ONCMY	NA	EST: CA388556	NA
	Pan troglodytes	PANTR	NA	Chromosome 15 (prediction based on NCBI build 1.1)	NA
	Phytophthora sojae	PHYSO	NA	EST: BE585238, CF841845, CF860858	NA
	Pongo pygmaeus	PONPY	NA	EST: CR547617, CR763766, CR762931	NA
	Rattus norvegicus	RAT	NA	Chromosome 7 (prediction based on NCBI build 3.1, RGSC v3.4)	NA

Table 1. Continued

Taxon	Species	Code	Strain	ID[a]	Gene
	Strongylocentrotus purpuratus	STRPU	NA	XP_789061 (GI:72047182) incomplete	NA
	Sus scrofa	PIG	NA	EST: BP153340, CF366264, CF364846, BI337006, CJ016803	NA
	Poephila guttata	POEGU	NA	EST: CK306898	NA
	Tetraodon nigroviridis	TETNG	NA	Chromosme 19 (genome v7.0)	NA
	Ustilago maydis	USTIMA	521	Q4PEJ8 (contig 52, scaffold 3, assembly 1), Q4PBY4 (contig 83, scaffold 4, assembly 1)	NA
	Xenopus tropicalis	XENTR	NA	EST: CR560067, AL874410	NA

[a]Swiss-Prot IDs appear in red, EMBL EST IDs in green, Genbank IDs in pink, RefSeq IDs in blue, chromosome entries in black, and others in magenta.
NA, not available.
DOI: 10.1371/journal.pcbi.0010063.t001

Stealth and Protists

Although higher eukaryotes haven't yet been investigated for the presence of phosphoglycan structures similar to the CPSs, such structures have been identified in *D. discoideum* and in *Leishmania* species. In *D. discoideum* such polysaccharides were found on lysosomal cysteine proteinases and spore coat proteins [36,37]. The lysosomal enzymes of *D. discoideum* have two types of carbohydrate modifications [38,39] found in two separate sets of lysosomal vesicles [40,41]. The major component of *Leishmania* lipophosphoglycan is a heteropolymer of 10–40 phosphodiester-linked disaccharide units, depending on species and developmental stage [42]. Lipophosphoglycan is predominantly expressed by promastigotes, is essential for intracellular survival in macrophages and for the virulence of *Leishmania major* and *L. donovani*, and disappears when the pathogen intracellularly differentiates into amastigotes within host phagolysosomes [43–47]. The genes encoding these hexose-phosphoryl transferases have been identified neither in *D. discoideum* nor in *Leishmania*. Given, however, Stealth's presumed enzymatic activity and its comparative biochemical characterization from three different *Leishmania* species using synthetic acceptor substrate analogs [48], the two Stealth proteins found in *Leishmania* and those found in *D. discoideum* are good candidates for this function.

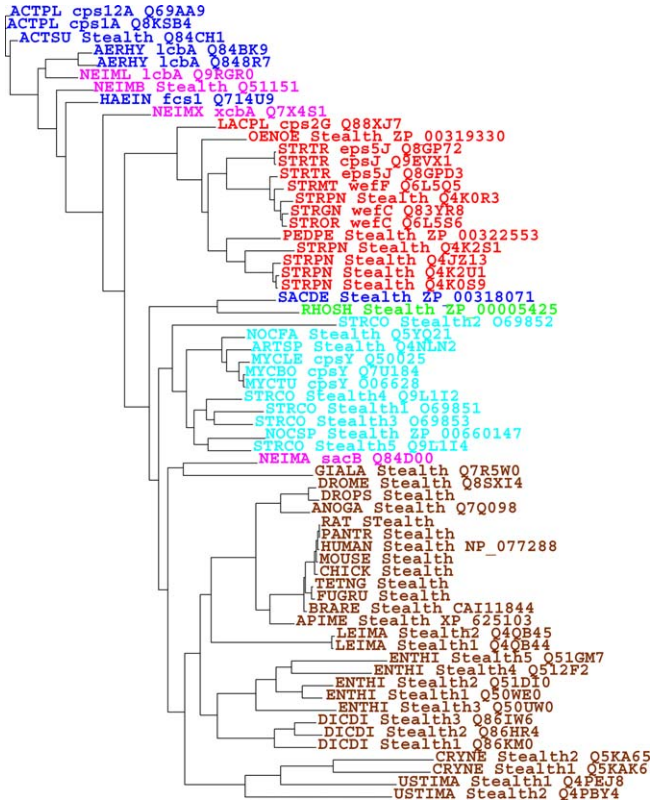

Figure 3. Phylogenetic Tree

Trees were calculated from amino acid sequence alignments of the four CRs. As in Figure 1, sequences are identified by a species code (see Table 1), protein name (from literature as proposed in this paper), and database accession number, and are color-coded. Dissimilarities are represented by the length of the branches (all with posterior probabilities above 0.95).
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Evolution of Stealth

The peculiar taxonomic distribution of Stealth (Figure 3) could be the outcome of two different evolutionary scenarios: (i) differential loss of an ancient protein already present in an ancestral form of life, or (ii) horizontal gene transfer between eukaryotes and eubacteria. The second hypothesis appears to be the more plausible, but the direction of the transfer is more difficult to assess. Overall, the protein tree largely follows species phylogeny, at least with regard to the higher level taxonomic groups. This indicates that transfer between eukaryotes and prokaryotes must have been an ancient event. However, several observations suggest that Stealth proteins continue to be horizontally transferred within and between certain bacterial groups. In Gram-negative bacteria, Stealth is inserted into group II capsule operons, which exhibit strong sequence similarity across many species, thus facilitating horizontal gene transfer via homologous recombination [49,50]. Moreover, certain Stealth genes have significantly lower G+C content than the remaining part of the genome [19,21,24,51], which is indicative of a recent acquisition from another species, and some of these genes are flanked by recombination-promoting IS insertion elements or residual fragments thereof [21,24].

Materials and Methods

Sequence analysis. Multiple amino acid sequence alignments of the four CRs were generated using T-Coffee [52]. The signal peptides were predicted with SignalP v2.0 using the combined NN/HMM-based method [53,54], the transmembrane predictions were made using TMHMM v2.0 [55,56], and the Lin-12/Notch repeats were identified using the profile PS50258 in PROSITE [57]. The EF-hand domains were detected using the Pfam HMM PF00036 [58].

The human and the fly gene structures were constructed with the aid of the trome database [59–61].

Sequence database searches. Other members of the Stealth protein family were identified by searching with either the human or the *Streptomyces coelicolor* CR2 using BLAST [18] on either nucleic acid or protein databases.

Calculation of sequence trees. For each CR a separate multiple amino acid sequence alignment was generated. These multiple alignments were concatenated, resulting in a multiple alignment that represents the four CRs. CRs that are absent in certain species are represented as gaps in the multiple alignment. Processed alignments were used to derive tree topologies using Bayesian inference of phylogeny as implemented by MrBayes v3.0 [62,63]. MrBayes was used with four heated chains over 200,000 generations, sampling every 20 trees. The likelihoods of these trees were examined to estimate the length of the burn-in phase, and all trees sampled 20,000 generations later than this point were used to create a consensus tree using the 50% majority rule. MrBayes was used with the mixed model of amino acid substitution, assuming the presence of invariant sites and using a gamma distribution approximated by four different rate categories to model rate variation between sites, estimating amino acid frequencies from the alignment. The consensus tree was displayed using DRAWGRAM of the PHYLIP package [64].

Supporting Information

Figure S1. Taxonomic Distribution of Stealth in Bacteria

Found at DOI: 10.1371/journal.pcbi.0010063.sg001 (57 KB DOC).

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Competing interests. The authors have declared that no competing interests exist.

Author contributions. PS, CDS, PB, and OZ conceived and designed the experiments. PS and CDS performed the experiments. PS, CDS, PB, and OZ analyzed the data and wrote the paper. ∎

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